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Award Number: W81XWH-12-1-0329

TITLE: Investigating an Evolutionarily Conserved Role for the Tousled-like Kinase in Genome Stability and as a Novel Target for the Treatment of Ovarian Cancer

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REPORT DATE: October 2013

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2013	Annual	30September2012–29September2013

4. TITLE AND SUBTITLE

Investigating an Evolutionarily Conserved Role for the Tousled-like Kinase in Genome Stability and as a Novel Target for the Treatment of Ovarian Cancer

5a. CONTRACT NUMBER
W81XWH-12-1-0329

5b. GRANT NUMBER
W81XWH-12-1-0329

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Texas MD Anderson Cancer Center
Houston, Texas 77030-4009

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command

Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

In this project we are utilizing *C. elegans* and normal and cancer-derived human cell lines to interrogate the role of the Tousled-like kinase family in mitotic spindle dynamics. Thus far, our *C. elegans* studies support our hypothesis that phosphorylation of TLK-1 by the CHK1 and Aurora B kinases promote the localization of TLK-1 to mitotic chromosomes where it facilitates spindle assembly and chromosome congression. In addition, we discovered that TLK-1 has an unexpected role in mitotic spindle positioning in the early *C. elegans* embryo. Since spindle positioning is dependent on microtubule-based mechanisms, this finding further supports our contention that TLK-1 is a microtubule-regulatory protein. In our human cell studies, we discovered that depletion of human Tlk1 has a dramatic effect on mitotic spindle assembly and microtubule behavior in a cancer cell line. Furthermore, human Tlk1 may also functionally inhibit the epithelial to mesenchymal transition (EMT). Both of these findings are extremely exciting in that human Tlk1 has an essential role in mitotic spindle assembly but also appears to regulate microtubule dynamics and the cytoskeleton more broadly. A role for Tlk1 in preventing EMT has never been reported and opens up an entirely new avenue of inquiry into this multi-faceted kinase family.

15. SUBJECT TERMS

Tousled-like kinase, C. elegans, Kinetochore, Spindle Assembly Checkpoint, Genome stability

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	20	19b. TELEPHONE NUMBER *(include area code)*

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INTRODUCTION:

The Tousled-like kinases comprise an evolutionarily conserved family of serine/threonine kinases that have been implicated in chromatin assembly and resistance of human cells to DNA damage (*1*). Our lab made the novel discovery that the *C. elegans* Tousled-like kinase TLK-1 is also essential for mitosis. We found that TLK-1 is a dynamic component of the outer kinetochore that integrates Aurora B and CHK1 signaling to affect microtubule-kinetochore interactions. Given that human TLK1 physically interacts with outer kinetochore components and is required for mitotic chromosome segregation (*2, 3*), we hypothesize that TLK1 has an analogous, conserved role in human cells. Furthermore, we propose that TLK1 inhibition will sensitize ovarian cancer cells to Paclitaxel- and Cisplatin-induced cell death. Our aims are to: 1) Assess the functional contribution of TLK-1 kinase activity and Aurora B- and Chk1-dependent TLK-1 phosphorylation to kinetochore-microtubule dynamics and cell cycle progression in *C. elegans* embryos; 2) Elucidate the mechanistic role of human TLK1 at the outer kinetochore and the impact of TLK1 kinase activity and Aurora B- or CHK1-dependent phosphorylation on the spindle assembly checkpoint in human cells. 3) Determine the consequences of silencing TLK1 on the sensitivity of a panel of ovarian cancer cell lines to Paclitaxel and Cisplatin.

BODY:

Aim 1: Assess the functional contribution of TLK-1 kinase activity and Aurora B- and CHK1-dependent TLK-1 phosphorylation to kinetochore-microtubule dynamics and cell cycle progression in *C. elegans* embryos.

The *C. elegans* Tousled-like kinase (TLK-1) is phosphorylated by the CHK-1 and Aurora B kinases at distinct sites (Threonine 610 and Serine 634 respectively) (Fig. 1) (*4, 5*). Notably, while immuno-staining with TLK-1 antibodies revealed that TLK-1 is localized to interphase and prophase nuclei, as it is in human cells (*6, 7*), phospho-specific antibodies recognizing pTLK-1(T610) or pTLK-1(S634) showed that both TLK-1 phospho-isoforms are localized to kinetochores and kinetochore-microtubules (*4, 5*). Further experiments revealed that both phospho-proteins are associated with the outer kinetochore and are downstream of BUB-1 and CENP-F in the kinetochore assembly hierarchy (*5*). This intriguing localization led to the hypothesis that Aurora B- and CHK1-dependent phosphorylation is required for the recruitment of TLK-1 to the outer kinetochore, where it has an essential role in the regulation of mitotic spindle dynamics and cell cycle progression.

Task 1.A- Creation of *C.elegans tlk-1* transgenic lines

To test our hypothesis, our first task was to create several transgenic constructs to assess the contribution of TLK-1 and Aurora B and CHK1-mediated phosphorylation to *C. elegans* embryogenesis (Table 1). For each transgene, the 5' 400 nucleotides of the full-length *tlk-1* cDNA were modified to be resistant to RNAi-mediated silencing by double-stranded (ds) RNA specific to this region of the wild type *tlk-1* cDNA (*tlk-1-500(RNAi)*)(Fig. 1). Changes were made in the third nucleotide of appropriate codons to ensure that the TLK-1 amino acid sequence was not altered. Site-directed mutagenesis was utilized to create phospho-mutant (alanine; T610A and S634A) and phospho-mimic (glutamic acid; T610E and S634E) residues in individual transgenes. Each transgene was fused at the 5' end with a GFP cDNA to create N-terminal in-frame GFP-fusion proteins. Transgene expression was placed under the control of the *pie-1* promoter and 3' UTR, which provide reliable expression in the germ line and early embryo (*8*). All constructs harbored a wild type *unc-119* gene, which was used to screen for transgene insertion following biolistic bombardment-mediated transformation of *unc-119* mutant hermaphrodites (*8*). Using this method, we successfully generated multiple *unc-119* rescued lines per transgene (Table 1 and data not shown). An RNAi-resistant transgene harboring a kinase dead mutation in the TLK-1 kinase domain (histidine 682 to arginine) is currently under construction (Fig. 1).

In our original proposal, we planned to utilize the Mos-TIC (Mos1 excision-induced transgene-instructed gene conversion) transgenesis method (*9*), but we were unable to generate *unc-119* rescued animals. Hence, we

switched to biolistic transformation, a method that results in low-copy single integration events and we have successfully used in the past (*8, 10*).

The *unc-119*-rescued transgenic lines were assessed for GFP expression using live microscopy and immunostaining with a GFP-specific antibody. For the lines listed in Table 1, GFP was visible in live animals at the 3-fold stage of embryogenesis (just before hatching) and by GFP-specific immunofluorescence in young embryos (data not shown). The lines were all viable, but those harboring *tlk-1* mutant transgenes had substantially reduced fecundity, suggesting that these mutant TLK-1 proteins may interfere with germ line development, gametogenesis, or fertilization (Fig. 2). Since animals harboring a deletion in the *tlk-1* locus are sterile (*11*), we plan to compare the germ line and fertility phenotypes in this strain with the *tlk-1* transgenic strains listed in Table 1.

Task 1.B- Evaluation of transgenic rescue of *tlk-1(RNAi)* and *tlk-1* mutant phenotypes

Depletion of TLK-1 via RNAi leads to 100% embryonic lethality (*4, 6*). To assess whether our TLK-1 transgenic lines could rescue the penetrant embryonic lethality of *tlk-1-500(RNAi)*, wild type L4 larvae and L4 larvae from each line were subjected to *control* and *tlk-1(500)RNAi* (delivered by feeding dsRNA-expressing bacteria)(*12*) for 24-48 hours at 20^{o}C. Embryonic viability was assessed (N=10 hermaphrodites/strain) and adult animals were fixed and stained with DAPI as well as tubulin and TLK-1-specific antibodies (*6*). As expected, the progeny of wild type non-transgenic hermaphrodites treated with *control(RNAi)* where fully viable, while *tlk-1-500(RNAi)* resulted in approximately 80% embryonic lethality (Fig. 3). All of the transgenic lines also displayed full viability on *control(RNAi)* and no significant differences in the extent of embryonic lethality compared to wild type hermaphrodites when treated with *tlk-1-500(RNAi)* (Fig. 3). These results indicate that under these conditions, none of the GFP-TLK-1 transgenic proteins are capable of rescuing the embryonic lethality of TLK-1 depleted embryos.

To determine whether any of the GFP-TLK-1 transgenes affected the cellular phenotype of *tlk-1-500(RNAi)* embryos, the fixed samples described above were subjected to deconvolution microscopy. Wild type embryos treated with *tlk-1-500(RNAi)* accumulate large, puffy nuclei that are substantially depleted of endogenous TLK-1 and appear to exit the cell cycle (Fig. 4, rows 1 & 2). In contrast, *tlk-1-500(RNAi)* embryos expressing the wild type GFP-TLK-1 transgene are similar to *control(RNAi)*-treated embryos at equivalent stages of embryogenesis (Fig. 4, rows 3 & 4); they do not accumulate the characteristic puffy nuclei of TLK-1 depleted embryos. Furthermore, the transgenic TLK-1-WT protein remains localized to interphase and prophase nuclei, as is endogenous TLK-1.

tlk-1-500(RNAi) embryos from the TLK-1(T610A) transgenic line appear to be more severely affected than wild type *tlk-1-500(RNAi)* embryos, with much larger nuclei appearing at earlier stages of embryogenesis (Fig. 4., rows 5 & 6). In addition, endogenous TLK-1 and TLK-1(T610A) do not accumulate in interphase or prophase nuclei in *control(RNAi)* embryos. Since Tousled-like kinases can oligomerize (*13*), the mutant transgenic protein may prevent the wild-type kinase from localizing appropriately. Like the TLK-1-WT transgenic strain, the TLK-1(T610E) strain appears to be partially resistant to *tlk-1-500(RNAi)* (Fig. 4., rows 7 & 8). However, both *control* and *tlk-1-500(RNAi)*-treated TLK-1(T610E) embryos display defects in metaphase chromosome alignment, suggesting that TLK-1(T610E) expression interferes with this process. In addition, the TLK-1(T610E) protein is clearly associated with metaphase chromosomes (Fig. 4., row 8, arrowhead) and may also recruit endogenous TLK-1 to metaphase chromosomes (Fig. 4., row 7, arrowhead). Together, these results support our hypothesis that phosphorylation of TLK-1(T610) occurs in the vicinity of kinetochores or recruits TLK-1 to kinetochores, where TLK-1 has a role in mitotic spindle assembly.

Like TLK-1(T610), modification of TLK-1(S634) also appears to differentially affect the *tlk-1-500(RNAi)* phenotype. TLK-1(S634A) transgenic embryos treated with *tlk-1-500(RNAi)* display a more severe phenotype than TLK-1(S634E) embryos under the same RNAi conditions (Fig. 4, rows 10 & 12). Both transgenic proteins

localize to interphase and prophase nuclei along with endogenous TLK-1 (Fig. 4, rows 9-12). In addition, the TLK-1(S634E) line appears to retain TLK-1 in metaphase cells (Fig. 4, row 11, arrow).

Task 1.C- Cell biological characterization of mitosis and kinetochore dynamics in GFP-TLK-1 transgenic lines

The results of Task 1 (A&B) support our hypothesis that phosphorylation of TLK-1 at T610 and S634 affect the localization and function of the TLK-1 kinase. We are know poised to further characterize these lines via live imaging to assess cell cycle progression and mitotic spindle dynamics in real time. In addition, we will utilize antibodies for different kinetochore and mitotic spindle associated proteins to assess the effect of these transgenic proteins on the localization of critical mitotic regulators and effectors.

To begin our live imaging experiments, we obtained a *C. elegans* line that expresses GFP-γ-tubulin and GFP-Histone H2B (*14*). We treated this line with *control* and *tlk-1(RNAi)* and imaged these embryos via spinning disc confocal microscopy as they progressed through the first mitotic division.

In *C. elegans*, the first embryonic division is asymmetric, culminating in a larger anterior blastomere and a smaller posterior daughter cell. This difference in size is determined by the position of the mitotic spindle, which ultimately defines the cleavage plane during cytokinesis (*15*). The anteroposterior (AP) axis of the one-cell embryo is determined at fertilization by the sperm entry point, which demarcates the posterior pole of the embryo (*16*). Upon sperm entry, the anteriorly-localized maternal nucleus undergoes two meiotic divisions to create the haploid maternal pronucleus, which is followed by the decondensation of the sperm to form the male pronucleus (*17*). Concomitantly, the sperm-donated centrioles duplicate, recruit pericentriolar material, and begin to nucleate mitotic spindle microtubules (*18*). The maternal pronucleus migrates toward the male pronucleus and the two pronuclei meet in the posterior half of the embryo. After pronuclear meeting (PNM), the (pro)nuclear-centrosome complex migrates anteriorly (centration) while rotating 90° to align along the AP axis (rotation). Differential cortical pulling forces acting on the astral microtubules emanating from each centrosome promote rotation (*19*). As the mitotic spindle rotates, nuclear envelope breakdown occurs, followed by kinetochore-microtubule-attachment and metaphase chromosome alignment perpendicular to the AP axis. At anaphase onset, the spindle elongates and undergoes posterior displacement, ultimately resulting in an asymmetric division.

In both *control* and *tlk-1(RNAi)* embryos, pronuclear migration, PNM, and centration occurred in a timely fashion. Likewise, there was no significant difference in the migration rate or positioning of the maternal pronucleus during migration or centration (Fig. 5 and (*20*)). In addition, the average timing of PNM relative to NEBD in *control* or *tlk-1(RNAi)* embryos was not significantly different, nor was the timing of metaphase chromosome alignment or anaphase onset (Fig. 5). However, in 47% of *tlk-1(RNAi)* embryos (n=15), there was a significant delay in mitotic spindle rotation (Fig. 5). In *control(RNAi)* embryos, rotation occurred prior to or during NEBD, whereas in the affected *tlk-1(RNAi)* embryos, rotation was delayed until after NEBD, and often occurred after metaphase chromosome alignment (Fig. 5). However, in all embryos examined, the mitotic spindle eventually rotated to lie along the anterior-posterior axis prior to the completion of anaphase (Fig. 5). Further analysis of the striking phenotype revealed that centrosome behavior is significantly altered in *tlk-1(RNAi)* embryos compared with controls (data not shown and (*20*)). Our previous analysis revealed that TLK-1 is required for the localization of Clasp/CLS-2, a microtubule-binding and regulatory protein, to the outer kinetochore (*5*). Interestingly, CLS-2 depleted embryos also display a rotation defect similar to *tlk-1(RNAi)* embryos (*21*). These results suggest that Clasp/CLS-2 may be a critical downstream effector of the TLK-1 kinase. Whether Clasp is a direct substrate or binding partner of the TLK-1 kinase is an interesting question that we will explore in *C. elegans* and human cells in the future.

Aim 2: Elucidate the mechanistic role of human TLK1 at the outer kinetochore and the impact of TLK1 kinase activity and Aurora B- or CHK1-dependent phosphorylation on the spindle assembly checkpoint in human cells.

Task 2.A- Quantitation and sub-cellular localization of TLK1 in human cell lines

Since the amino acid sequence of Tousled-like kinases is highly conserved, we assessed whether the localization of TLK-1 to the outer kinetochore that we observed in *C. elegans* is conserved in human cells. Hence, we fixed normal breast epithelial cells (MCF10A) and immunostained with a commercially available hTLK1 antibody (Cell Signaling, Danvers, MA) and a tubulin-specific antibody (Sigma, St. Louis, MO). This experiment revealed strong nuclear staining in interphase and prophase cells (as previously reported (*7*)) (Fig. 6). We also noted a number of microtubule-associated foci in mitotic cells (data not shown). To ascertain whether this staining is specific for human Tlk1, we sought to reduce Tlk1 via shRNA targeting (as proposed in Task 2B).

Task 2.B- Creation of GFP-TLK1-expressing cell lines and shRNA analysis

To deplete Tlk1 from human cells, we obtained two different hTlk1 short hairpin RNAs (shRNAs) and a scrambled control shRNA from the MDACC shRNA core. All shRNAs are in the GIPZ Lentiviral vector (ThermoScientific, Pittsburgh, PA). Lentivirus was produced in 293T cells and MCF10A cells were infected with each virus. Cells were fixed 2, 3, 4 and 5 days post-infection and stained with DAPI, and Tlk1 and tubulin-specific antibodies (Fig. 6). Both Tlk1 shRNAs resulted in a significant reduction in nuclear Tlk1 immunostaining at day 2 post-infection (Figs. 6 and 7). However, in cells treated with shRNA#1, Tlk1 expression returned to control levels by day 3 and was retained in day 5 post-infection cells (Figs. 6A and 7). In contrast, Tlk1 expression remained low in cells treated with Tlk1shRNA#2 (Figs. 6B and 7). Interestingly, by day 4 post-infection, the morphology of MCF10A cells treated with shRNA#2 was strikingly different from control-treated cells. The nuclei were elongated and the cells developed a spindly appearance (Fig. 6B). These changes are reminiscent of cellular transformation with oncogenic Ras (*22*), and may indicate a transition from an epithelial to mesenchymal identity (EMT)(*23*).

In addition to MCF10A cells, we also treated a Triple Negative Breast tumor cell line, MDA486, with the scrambled control shRNA and Tlk1shRNA#1. This resulted in significant depletion of Tlk1 throughout the 5-day infection period (Fig. 7, right columns). Strikingly, Tlk1 loss resulted in the formation of large polyploid nuclei and mono-polar spindles (Fig. 8), as well as wavy cytoplasmic microtubule bundles (Fig. 8). These results are consistent with Tlk1 having a critical role in mitotic spindle assembly and microtubule dynamics.

As described above, our progress on this Task has led to several intriguing findings. To further interrogate Tlk1 function in human cells, we plan to generate lentivirus against three additional Tlk1 shRNAs and compare the efficacy of all five Tlk1 shRNAs in reducing Tlk1 expression in MCF10A, MDA486, and multiple ovarian cancer cell lines. If we consistently find EMT like changes, we will assay a variety of EMT specific markers to confirm whether EMT is occurring in the absence of Tlk1. In addition, we will further assess the nature of the microtubule bundles in MDA486 cells and whether Clasp and other microtubule-regulatory proteins are involved in their formation. As described in the original Statement of Work (SOW), we plan to create inducible Tlk1 shRNA cells lines with the two most effective Tlk1 shRNAs. These cell lines will be transfected with GFP-Tubulin and mCherry Histone H2B transgenes (a gift from K. Ullman, University of Utah) and the effect of Tlk1 shRNA induction on mitotic spindle dynamics and cell cycle progression will be assayed by spinning disc confocal microscopy of living cells. The localization of GFP-Tlk1 fusion proteins will also be addressed as described in the original SOW.

In the coming project period, we plan to continue the experiments described in Aims 1 and 2. Once we determine which Tlk1 shRNAs are most effective and create inducible lines, we will initiate the Paclitaxel and Cisplatin sensitivity assays described in Aim 3.

KEY RESEARCH ACCOMPLISHMENTS:

-Created RNAi-resistant TLK-1 wild type and mutant *C. elegans* transgenic lines

-Discovered that T610E and TS634E phospho-mimic transgenes partially rescue *tlk-1(RNAi)* while the corresponding phospho-mutant transgenes are more severely affected than wild-type embryos

-Discovered that *C. elegans* TLK-1 is required for timely mitotic spindle rotation and positioning

-Assessed the efficacy of two human Tlk1 shRNAs in depleting Tlk1 from human cell lines

-Discovered that loss of human Tlk1 leads to aberrant mitotic spindles and microtubule arrays in a human cell line

-Discovered that Tlk1 depletion may lead to an epithelial to mesenchymal transition in human epithelial cells, suggesting that Tlk1 may have tumor suppressive properties

REPORTABLE OUTCOMES:

The following manuscripts will be submitted within the next few months:

Ford, J.R., Adams, H.P., Furuta, T., and J.M. Schumacher. The Tousled-like kinase potentiates centrosome dynamics to ensure timely mitotic spindle rotation in the early C. elegans embryo. Manuscript in Preparation.

De Orbeta, J., Deyter, G.M.R., Furuta, T., and J.M. Schumacher. The C. elegans Tousled-like kinase is a CHK1 substrate that localizes to the outer kinetochore and is essential for CLASP recruitment. Manuscript in Preparation.

CONCLUSION:

During the first project period of this award we made significant progress on Aims 1 and 2 as detailed above. Here we combining the strengths of a genetic model system, *C. elegans*, with the biology of normal and cancer-derived human cell cultures to interrogate the role of the evolutionarily conserved Tousled-like kinase family in cell cycle progression and mitotic spindle dynamics. This kinase family has already been implicated in the DNA damage checkpoint and cisplatin sensitivity (*24-26*). However, we hypothesize that it has an additional conserved role in mitotic spindle assembly that may affect the sensitivity of cells to Paclitaxel and other drugs that target microtubule dynamics. Such a dual role and the "drugability" of kinases positions the Tousled-like kinase as an ideal candidate target for the treatment of ovarian cancer and other cancers that are treated with a combination of Cisplatin and Paclitaxel.

Thus far our studies in *C. elegans* support our hypothesis that phosphorylation of TLK-1(T610) and (S634) by the CHK1 and Aurora B kinases promote the localization of TLK-1 to mitotic chromosomes where it facilitates mitotic spindle assembly and chromosome congression. However, the inability of any of our *tlk-1* transgenes to rescue *tlk-1-500(RNAi)* lethality may be a consequence of using a heterologous promoter that doesn't replicate endogenous *tlk-1* expression patterns or could be due to some "leakiness" in our RNAi-resistant strategy, such that the transgenes may be partially targeted by *tlk-1-500(RNAi)*. To circumvent these problems we are exploring the use of Crispr/Cas9 genetic engineering to introduce the TLK-1(T610) and (S634) point mutations into the endogenous *tlk-1* locus. This technology is rapidly changing genetic approaches in various model

systems including mouse, *C. elegans*, and even human cultured cells (*27-30*). The advantage of using this strategy is that the mutants will be expressed under the same promoter and regulatory sequences as the wild type endogenous *tlk-1* gene. This strategy will also be useful for tagging both mutant and wild type *tlk-1* with GFP or other fusion proteins. Nevertheless, our current transgenic results support our hypothesis that phosphorylation of TLK-1 by Aurora B and CHK1 promote TLK-1's localization to the outer kinetochore where it is likely to play a critical role in mitotic spindle assembly and chromosome segregation.

In the course of this research we discovered that the Tousled-like kinase (TLK-1) has an unexpected role in mitotic spindle positioning in the early *C. elegans* embryo. Since spindle positioning is dependent on microtubule-based mechanisms, this finding also supports our contention that TLK-1 is a microtubule-interacting protein that regulates spindle dynamics. Furthermore, accurate spindle positioning is essential in stem cell niches and regulates cell fate in a variety of tissues (*31*). It will be very interesting to determine whether human Tlk1 also promotes spindle positioning and whether stem cell fates are affected by Tlk1 expression.

Aim 2 is focused on exploring the functional roles of Tlk1 in human cells. To this end we tested the ability of two distinct human Tlk1 shRNAs to deplete Tlk1 from normal and cancerous human cells. This analysis revealed that depleting Tlk1 has a dramatic effect on mitotic spindle assembly and microtubule dynamics in a human cancer cell line. Furthermore, our preliminary data suggest that human Tlk1 may also functionally inhibit the epithelial to mesenchymal transition (EMT). Both of these findings are extremely exciting in that human Tlk1 does indeed have an essential role in mitotic spindle assembly but also appears to regulate microtubule dynamics and the cytoskeleton more broadly. A role for Tousled in preventing EMT has never been reported and opens up an entirely new avenue of inquiry into this multi-faceted kinase.

In the coming year, we will continue our work on Aims 1 and 2, as well as complete the ground-work for and begin the experiments described in Aim 3, which are focused on a key question: Will depletion/inhibition of human Tlk1 sensitize ovarian cancer cell lines to Cisplatin and Paclitaxel?

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APPENDICES: Table 1 and Figures 1-8

Table 1: Transgenic *C. elegans* Strains Generated

Strain	Transgene	Localization	Rescue *tlk-1-500(RNAi)*
N2 –Wild type	N/A	Interphase, prophase nuclei, mitotic centrosomes	N/A
JS1239	GFP-*(rr)-tlk-1* WT	Interphase, prophase, telophase nuclei	Partial
JS1298	GFP-*(rr)-tlk-1 (T610A)*	Cytoplasm, interphase and prophase nuclei	No
JS1273	GFP-*(rr)-tlk-1 (T610E)*	Interphase, prophase nuclei, metaphase chromosomes	Partial, some metaphase defects in *control(RNAi)* cells.
JS1288	GFP-*(rr)-tlk-1 (S634A)*	Interphase, prophase nuclei	No
JS1296	GFP-*(rr)-tlk-1 (S634E)*	Interphase, prophase nuclei,	Partial

rr: RNAi-resistant; N/A: Not applicable

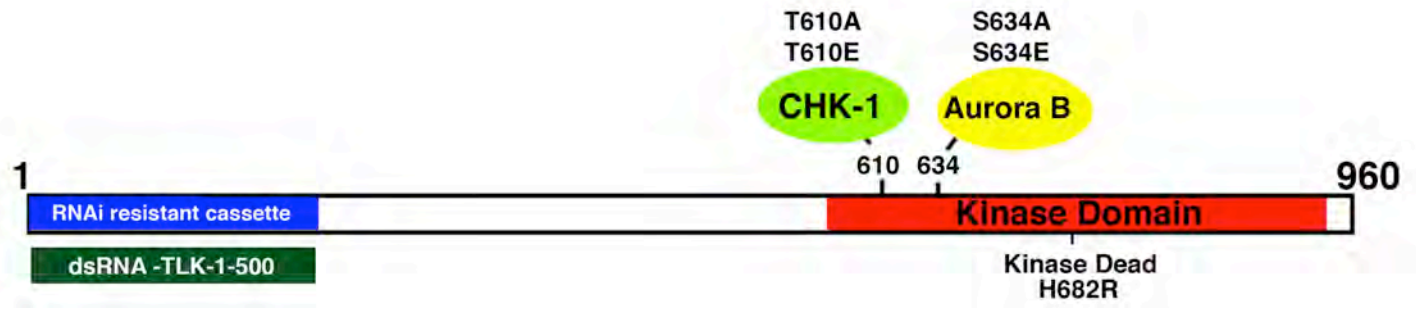

Figure 1: Design of *C. elegans* TLK-1 transgenes.
TLK-1 is a 960 amino acid protein with a C-terminal domain kinase. An oligonucleotide corresponding to an RNAi-resistant (rr) version of nucleotides 1-390 was commercially prepared, PCR amplified, and ligated to the remainder of the TLK-1 cDNA. The indicated point mutations were generated by PCR-based site-directed mutagenesis. Each construct was fused in frame with GFP at the N-terminus. All constructs were confirmed by DNA sequencing of the full-length GFP-TLK-1 transgene.

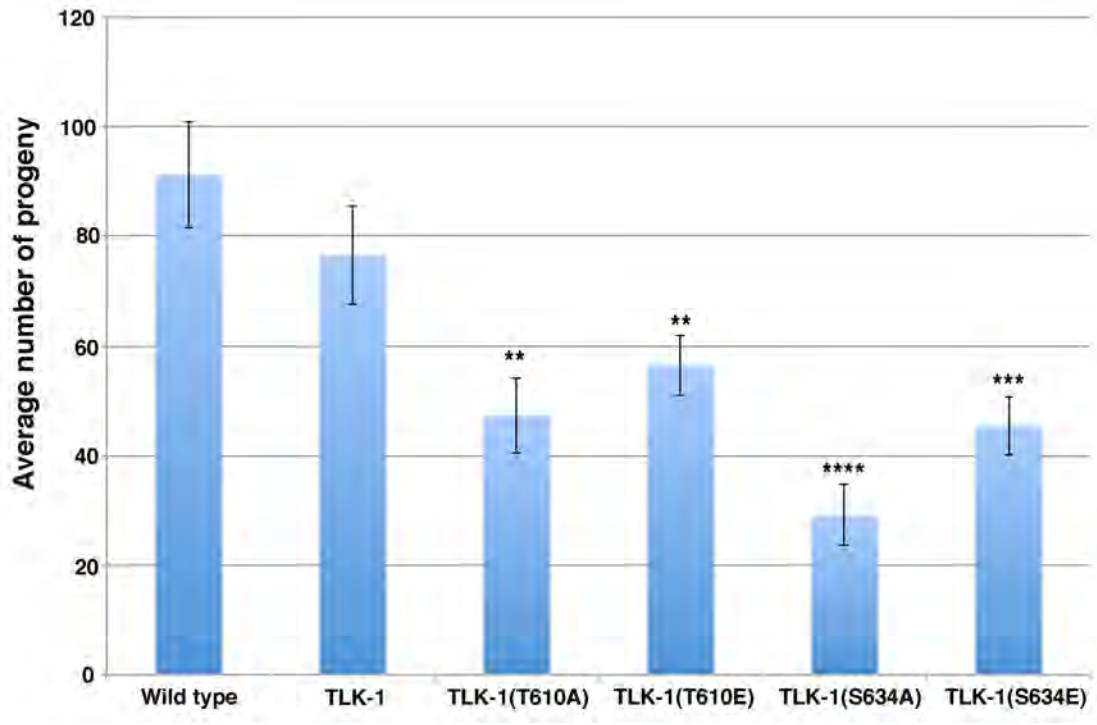

Figure 2: Number of progeny produced in 24 hours by wild type and TLK-1 transgenic lines. Individual wild type L4 hermaphrodite larva and L4 larva from the indicated lines were placed on individual plates seeded with OP-50 bacteria and incubated for 24 hours at 20°C. The adults were removed and the number of embryos on the plates counted. The mutant transgenic animals did not appear to be egg-laying defective but rather produced fewer embryos than wild type and animals harboring a wild type version of the GFP-TLK-1 transgene. Error bars represent standard error of the means. P values were calculated with respect to number of progeny/wild-type non-transgenic hermaphrodite. N=10 hermaphrodites/line. **- $0.01 < p < 0.05$; *** -$0.001 < p < 0.01$; ****-$0.0001 < p < 0.001$.

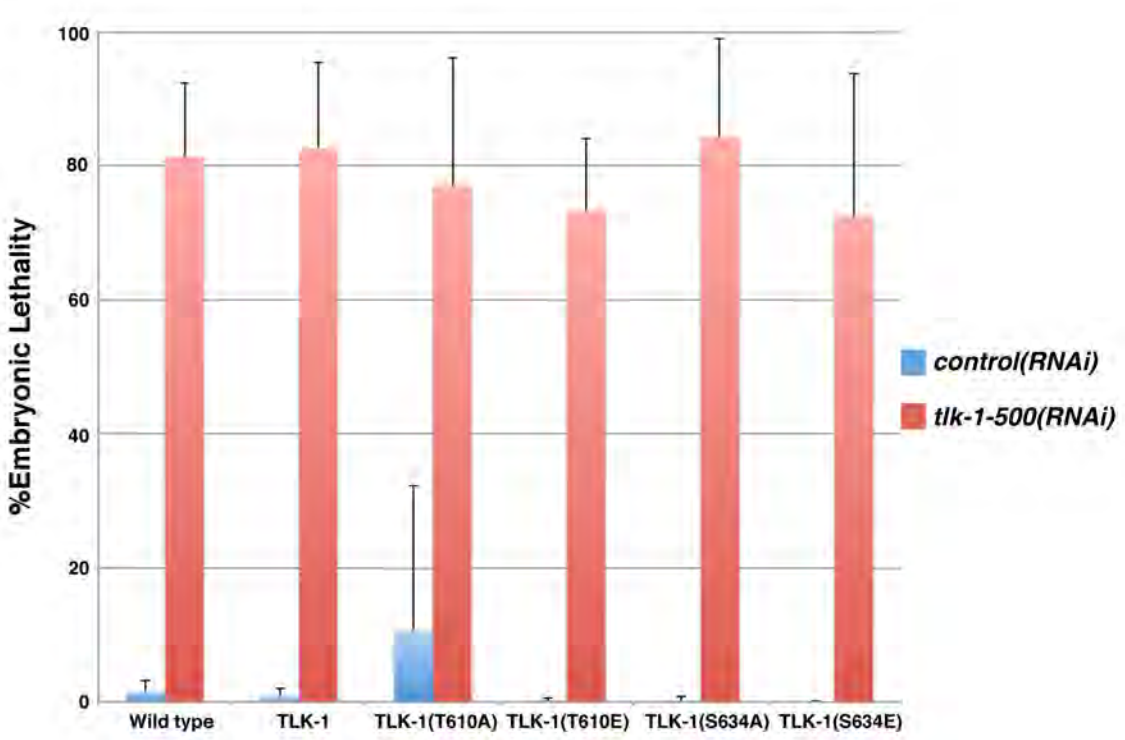

Figure 3: TLK-1 transgenes do not rescue *tlk-1(500)RNAi* embryonic lethality
Wild type L4 larva and L4 larva of the indicated transgenic lines (here and Table 1) were fed *control(RNAi)* or *tlk-1-500(RNAi*) bacteria. After 24 hours at 20°C, adults were removed and fixed for immunostaining (Fig. 4). The number of embryos on each plate were counted and compared to the number of hatched or unhatched embryos on the plates 24 hours later. N=progeny of 10 hermaphrodites assayed per strain. The average % embryonic lethality is shown. Error bars represent standard error of the means. $P > 0.05$ for all lines compared to wild type treated with the same RNAi.

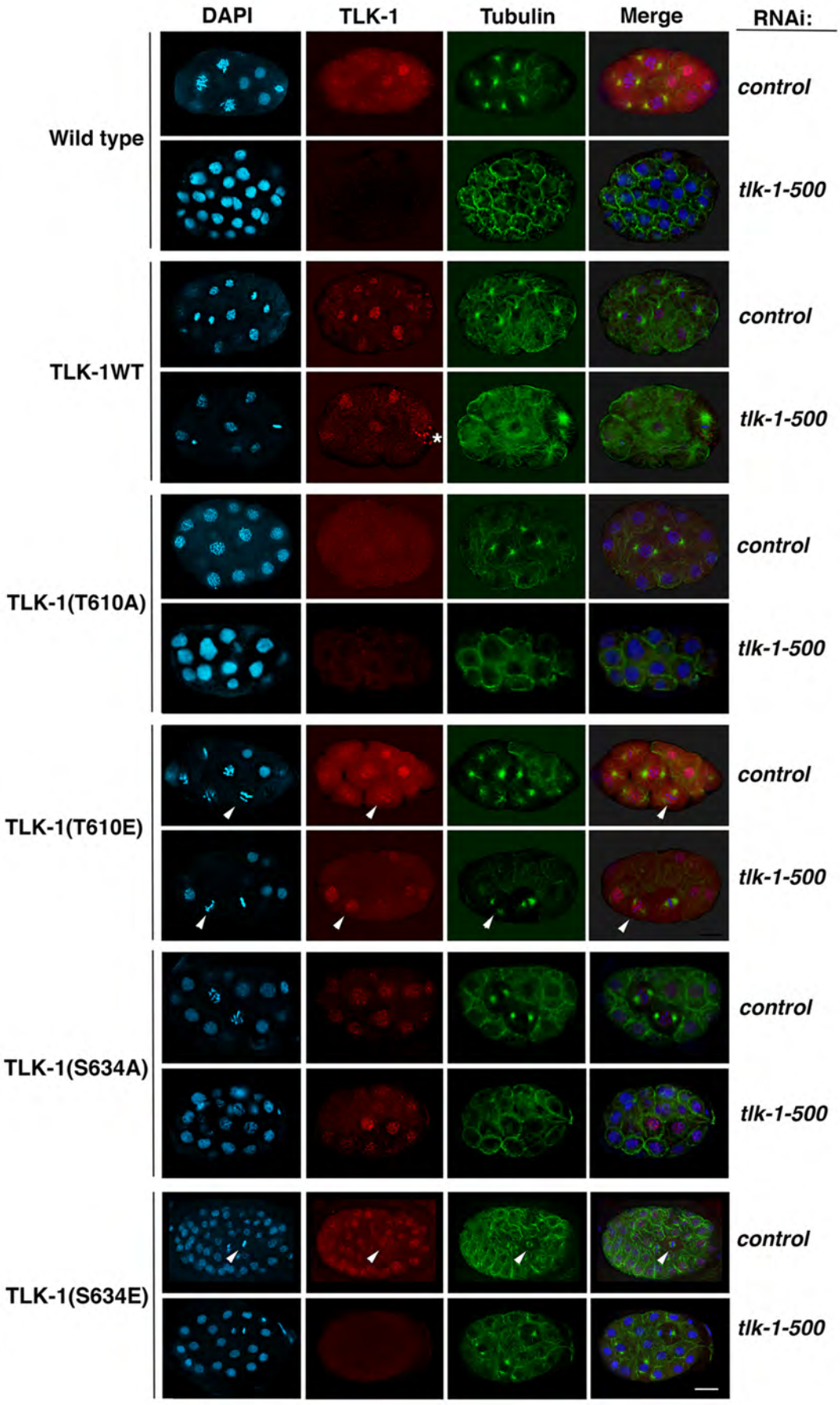
DAPI
TLK-1
Tubulin
Merge
RNAi:
Wild type
control
tlk-1-500
TLK-1WT
control
tlk-1-500
TLK-1(T610A)
control
tlk-1-500
TLK-1(T610E)
control
tlk-1-500
TLK-1(S634A)
control
tlk-1-500
TLK-1(S634E)
control
tlk-1-500

Figure 4 (previous page): *tlk-1(rr)* transgenes differentially affect the *tlk-1-500(RNAi)* phenotype
Wild type L4 larvae and L4 larvae from the indicated transgenic lines were fed *control(RNAi)* and *tlk-1-500(RNAi)* bacteria for 24 hours at 20^{o}C. The animals were gently pressed under a cover slip to release their embryos. The samples were fixed and stained with DAPI and TLK-1 and tubulin-specific antibodies. Scale bar = 10 μm.

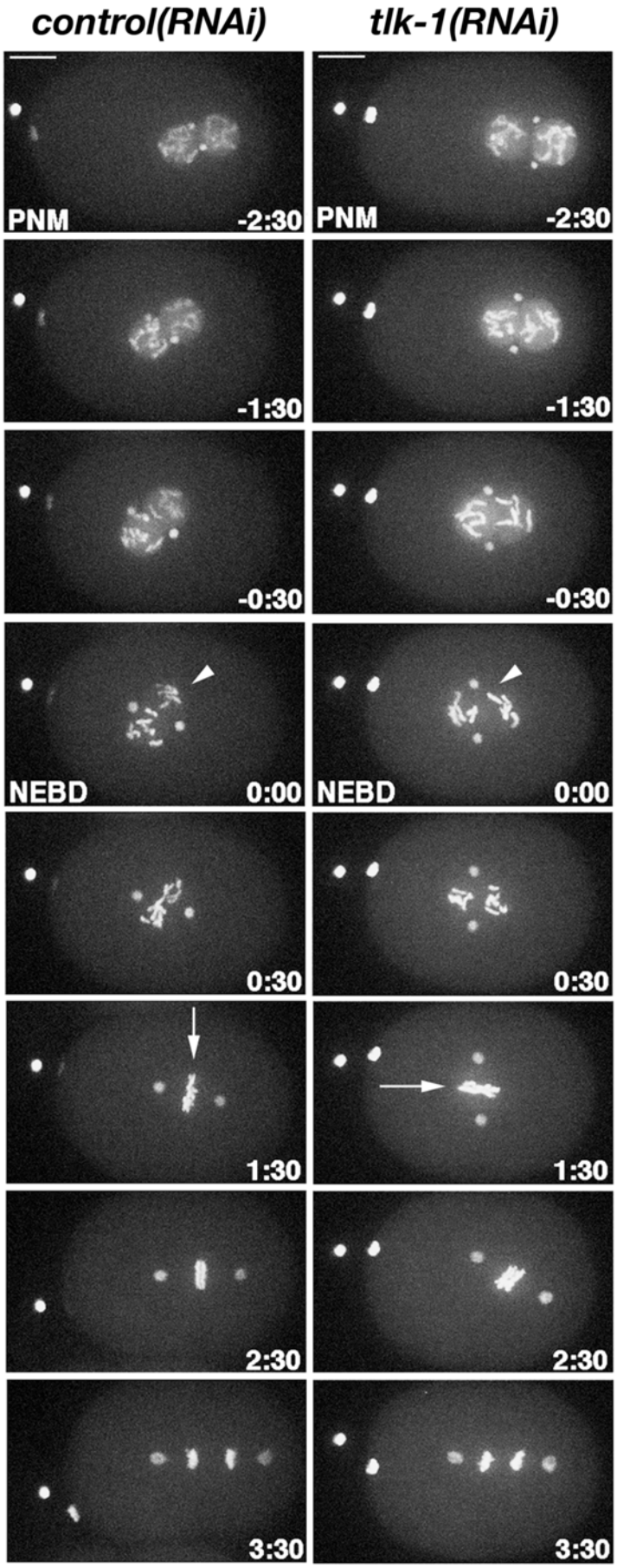

Figure 5. TLK-1 is required for timely mitotic spindle rotation in the one-cell *C. elegans* embryo

Embryos from transgenic hermaphrodites expressing GFP-γ-tubulin and GFP-Histone H2B treated with *control(RNAi)* or *tlk-1(RNAi)* for 24 hours at 25°C. Embryos were mounted and Subjected to live imaging via spinning disc microscopy. Time is from nuclear envelope breakdown. Arrows point to metaphase plates that are in the correct orientation (*control(RNAi))* and incorrect orientation (*tlk-1(RNAi)*).

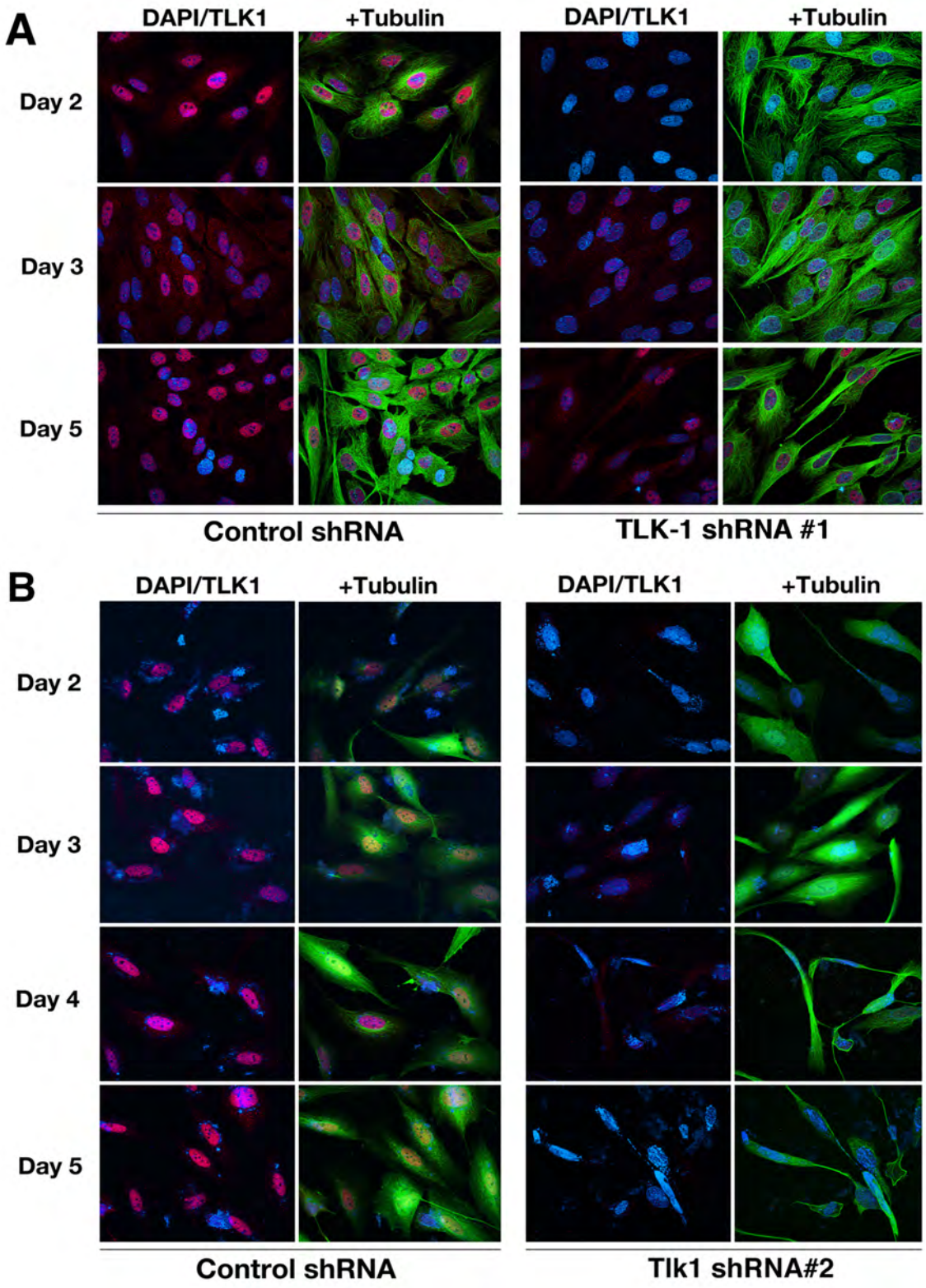

Figure 6: Tlk1 depletion in MCF10A cell changes cell morphology
MCF10A breast epithelial cells were infected with Lentivirus harboring a scrambled control shRNA or one of two distinct Tlk1-specific shRNAs (A, B). Cells were fixed 2, 3, 4, or 5 days after infection and stained with DAPI, and Tlk1 and Tubulin-specific antibodies. Note the changes in cell morphology changes in (B) 4 and 5 days post-infection with shRNA#2.

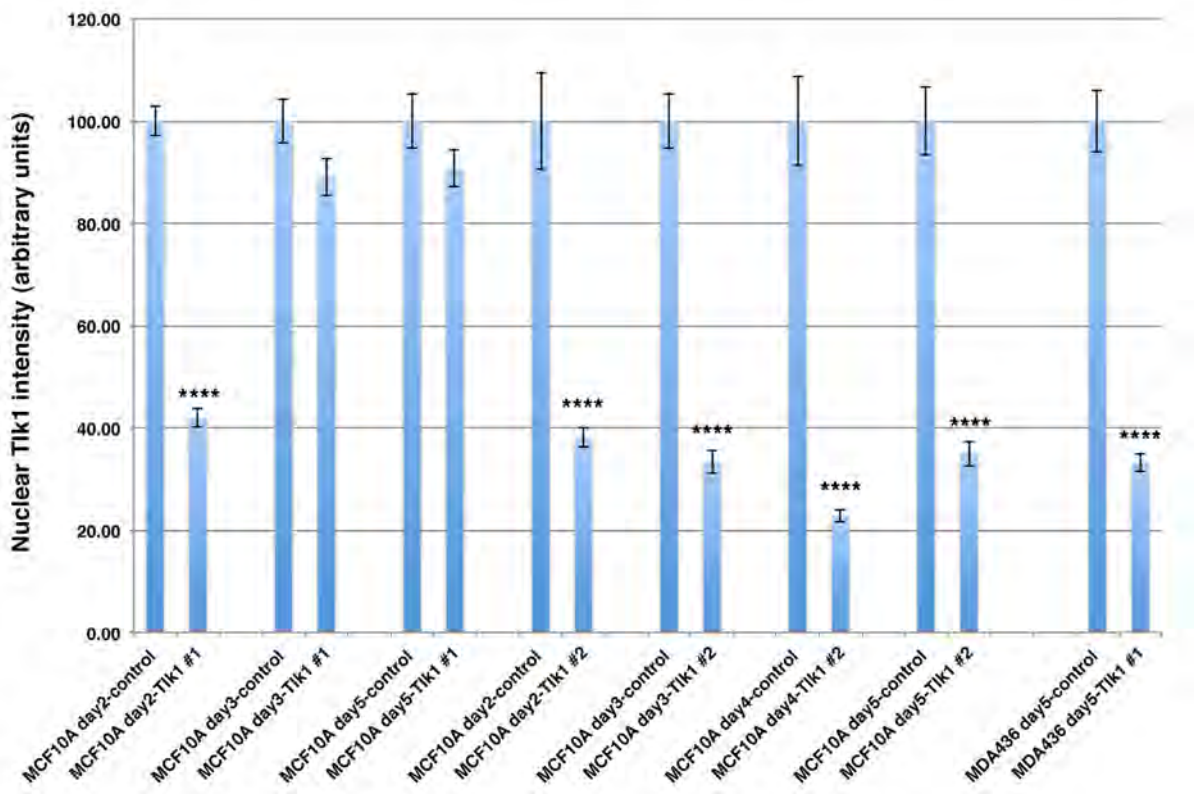

Figure 7: Quantitation of the efficiency of Tlk1 depletion by Tlk1-specific shRNAs
MCF10A and MDA486 cells were infected with lentiviruses harboring a scrambled control or Tlk1-specific shRNAs. Cells were fixed after 2, 3, 4, and 5 days-post infection and stained with DAPI and Tlk1 and tubulin-specific antibodies. Images were acquired using a Zeiss Apotome microscope and exposure times were identical between control and Tlk1 shRNA treated slides for each cell line. The mean nuclear TLK1 immunostaining was quantified using Image J software and control levels normalized to 100. Error bars represent standard error of the means. Approximately 100 nuclei were quantitated for each condition. ****: $p < .001$

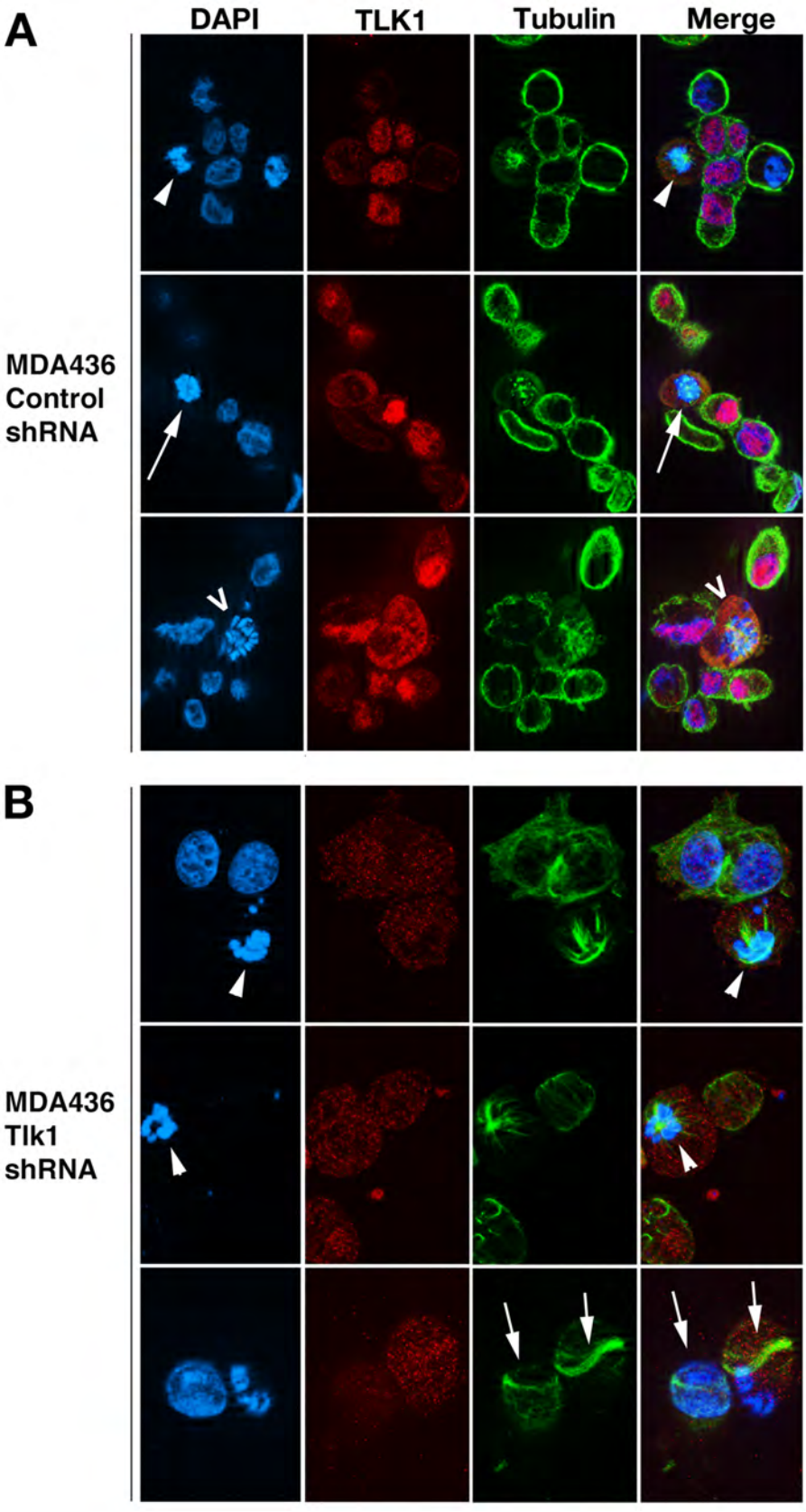

Figure 8: Tlk1 depletion result in mono-polar spindles and aberrant microtubule bundles in MDA486 cells
MDA486 breast cancer cells were infected with lentivirus harboring a scrambled control or Tlk1#1 shRNAs (see Fig. 7). A) Arrowhead and arrows point to mitotic cells. B) Arrowheads point to monopolar spindles. Arrows point to wavy microtubule bundles in multiple cells.